INTRACELLULAR DEGRADATION OF NEWLY SYNTHESIZED COLLAGEN IS CONFORMATION-DEPENDENT

Studies in human skin fibroblasts

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1. Introduction

Collagen, the most abundant extracellular protein, is a pre-requisite for the proper structure and function of most organs. Levels of collagen result from the synthesis and extracellular degradation by collagenase-mediated and/or phagocytic mechanisms and also from intracellular degradation of newly synthesized collagen. Hydroxyproline in collagen is formed by hydroxylation of proline residues which have been incorporated into peptide linkage [1]. After in vitro or in vivo labeling with [^{14}C]proline most of the hydroxy-[^{14}C]proline is found in collagen or its precursors. However, a significant amount rapidly appears in either free form or as small peptides in the medium of cultured cells [2–12] and organs [13–21] or is excreted in urine of mammals [22–24]. Although the rapid appearance of hydroxyproline in low molecular form was reported several years ago, it has been investigated systematically only recently [2–6,13] and recognized to be due to intracellular degradation of newly synthesized collagen [2,13].

We tested whether intracellular collagen degradation depends on the thermal stability of collagen and hence its triple helical conformation which is stabilized by the ascorbate-mediated hydroxylation of proline to hydroxyproline [1,25]. Experiments performed under various culture conditions, i.e., in the presence or absence of ascorbate or serum, at different temperatures or cell densities show that intracellular degradation is considerable, variable and indeed conformation-dependent and thus may play an important role in the regulation of tissue levels and the quality control of this secretory protein.

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2. Materials and methods

2.1. *Human skin fibroblasts*

Cells from a control were cultured and subcultured according to routine techniques in MEM and 10% fetal calf serum (FCS). The subcultivation numbers were between the 8th and 12th passages.

2.2. *Purification of [^{14}C]proline*

[U-^{14}C]Proline (New England Nuclear; 285 mCi/mmol) was purified on Dowex 50W-X8 (0.9 × 25 cm), lyophilized, dissolved in 50 mM Tris–HCl (pH 7.4) and used to label the cells.

2.3. *Labelling of cells*

Cells plated at different densities [26] in 80 mm dishes were preincubated for 16 h at 37°C with or without ascorbate (50 μg/ml), rinsed twice with PBS and incubated at the designated conditions (table 1) for 8 h in 5 ml MEM containing 50 μCi [^{14}C]proline.

2.4. *Quantitation of intracellular collagen degradation*

Collagen degradation was estimated by the amount of dialysable hydroxyproline per total hydroxyproline formed. Medium and cell layer were combined and sonicated. One-half was lyophilized, hydrolyzed (6 N HCl in N_2, 110°C, 24 h) and assayed directly for

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hydroxy-[^{14}C]proline (=total hydroxyproline formed). The other half of the sample was dialysed against 4 vol. 0.5 M acetic acid for 48 h at 4°C. The dialysate was lyophilised, hydrolysed and assayed for hydroxy-[^{14}C]proline. The total dialysable hydroxy-[^{14}C]proline was calculated after correcting for the volume of sample in the dialysis bag. The retentate was further dialysed and used for the estimation of prolyl hydroxylation of the collagenous proteins (below). [^{3}H]-Aspartic acid was added to all samples as internal standard. The labeled amino acids were separated by an automated amino acid analyzer (Biochrom). The losses of hydroxy-[^{14}C]proline in sample preparation were corrected based on the yield of [^{3}H]aspartic acid.

2.5. *Extent of prolyl hydroxylation in newly synthesized collagen*

To determine the extent of hydroxylation of prolyl residues in collagenous proteins, one aliquot of the retentate was digested with purified bacterial collagenase (Advance Biofactures, form III) [27], and undigested protein was precipitated with cold 5% trichloroacetic acid. The ratio of hydroxyproline to proline in the supernatant fluid was determined.

3. Results and discussion

In control experiments we first ascertained that hydroxylation of free proline to hydroxyproline did not occur because hydroxy-[^{14}C]proline was not produced in cultures labeled in the presence of cycloheximide (20 μg/ml). Also, the formation of hydroxy-[^{14}C]proline from bound prolyl residues was prevented by the simultaneous incubation of cells with [^{14}C]proline and α,α′-dipyridyl (0.5 mM). Furthermore, we found that the degradation of collagen did not occur extracellularly or by phagocytosis preceeding destruction.

(1) Isolated [^{14}C]procollagen added to control cultures was recovered intact and did not give rise to dialysable hydroxyproline.
(2) The proportion of dialysable hydroxy-[^{14}C]proline remained constant when the pulsed cultures were chased with [^{12}C]proline or treated with α,α′-dipyridyl or cycloheximide.

In a first set of experiments we found that at 37°C and in the presence of ascorbate, intracellular collagen degradation ranged from 13–44% (table 1). Similar values have been reported [2–6,9,10]. The amount of dialysable hydroxyproline was higher in sparse than in dense cultures (see also [5,6,9]) and equally so in the absence of serum. Here it correlated inversely with the extent of hydroxylation of collagen, which ranged from 45–22%.

Since the denaturation temperature of collagen has been shown to depend linearly on the degree of hydroxylation [25], we did the following two sets of experiments.

(i) To produce underhydroxylated collagen, cells were labeled in the absence of ascorbate. The extent of hydroxylation was 1/3rd (table 1).

Table 1

Expt	Culture conditions				Intracellular collagen degradation (%)[a]	Extent of prolyl hydroxylation of collagen (%)[a]
	Cell density	Temp. (°C)	Ascorbate (50 μg/ml)	Dialysed FCS (10%)		
I	Sparse	37	+	–	44	22
	Dense	37	+	–	23	32
	Sparse	37	+	+	29	28
	Dense	37	+	+	13	45
II	Dense	37	–	+	54	16
	Dense	30	–	+	51	15
	Dense	23	–	+	14	15
III	Dense	42	+	+	49	51
	Dense	37	+	+	14	43
	Dense	30	+	+	12	40

[a] Mean of 2 to 4 determinations

Degradation at 37°C and 30°C, i.e., above the melting temperature of underhydroxylated collagen was increased 4-fold. However, at 23°C, i.e., below its melting temperature, degradation was unchanged.

(ii) To produce fully hydroxylated collagen, cells were labeled in the presence of ascorbate. The extent of hydroxylation increased with temperature, from 40% at 30°C to 51% at 42°C, possibly because at the higher temperature helix formation was delayed and the procollagen chains had access to prolyl hydroxylase for longer. Degradation was low at 30°C and 37°C, i.e., below the melting temperature, but high at 42°C, i.e., above the melting temperature of fully hydroxylated collagen. These results corroborate and extend [2,5]. Following incorporation of proline analogues such as azetidine [2] or *cis*-4-hydroxyproline [5], defective collagen was produced, and thus intracellular degradation was markedly elevated.

Two general conditions have now been identified in which intracellular collagen degradation is significantly enhanced: increase of cyclic AMP content of cells [4]; and impairment of helical configuration of collagen. This can be caused by:

(i) Underhydroxylation of prolyl residues either during log-phase growth, or owing to the absence of ascorbate or after the substitution of analogues for proline;

(ii) Synthesis of collagen above its melting temperature.

Since the intracellular degradation of collagen is a rapid process [2,13] it may be an ideally suited mechanism by which cells modulate production and monitor quality of collagen prior to its secretion.

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